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CRYOPRESERVATION OF AMERICAN KESTREL SEMEN WITH DIMETHYLSULFOXIDE

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ABSTRACT.—Semen samples from 15 male American Kestrels (*Falco sparverius*) were frozen in dimethyl sulfoxide (DMSO). The semen was thawed 1–14 mo later and used to inseminate six females during three breeding seasons. Kestrels inseminated with thawed semen containing 4% DMSO produced only infertile eggs ($N = 14$). Kestrels inseminated with thawed semen containing 6%, 8%, or 10% DMSO produced fertile eggs ($N = 14$) and live chicks ($N = 6$). Progressive motility of spermatozoa in thawed semen containing 10% DMSO was less (44 ± 6%) than in thawed semen containing 6% (62 ± 10%) or 8% (61 ± 1%) DMSO.

Criopreservación del semen del Halcón Cernícalo (*Falco sparverius*) con dimetilsulfóxido

EXTRACTO.—Muestras de semen de 15 Halcones Cernícalo (*F. sparverius*) fueron congeladas en dimetilsulfóxido (DMSO). El semen fue descongelado entre 1 y 14 meses más tarde, y fue usado para inseminar seis halcones hembras durante tres períodos reproductivos. Las que fueron inseminadas con semen descongelado que contenía 4% de DMSO, solamente produjeron huevos estériles ($N = 14$); las otras que fueron inseminadas con semen descongelado que contenía 6%, 8% o 10% de DMSO produjeron huevos fértiles ($N = 14$) y pollos vivos ($N = 6$). La movilidad de espermatozoides en semen descongelado que contenía 10% DMSO fue menor (44 ± 6%) que en semen descongelado que contenía 6% (62 ± 10%) u 8% (61 ± 1%) DMSO.

[Traducción de Eudoxio Paredes-Ruiz]

Recent advances make it possible to collect and preserve sperm and to inseminate many mammals and some birds (Seager et al. 1978, Gee 1983). When genetic diversity is lost, frozen gene pools (gamete or embryo banks) can reestablish genetic diversity in captive or small native populations. The frozen material contains genetic variation of the original population from which the materials were collected. Methods are now available for the cryopreservation of semen from chickens (*Gallus domesticus*; Sexton 1980), Sandhill Cranes (*Grus canadensis*; Gee et al. 1985), and Aleutian Canada Geese (*Branta canadensis leucoparia*; Gee and Sexton 1990).

Many species of raptors are listed as endangered (Cade and Temple 1977, King 1978) and captive propagation and release programs have been successful with some (Weaver and Cade 1983). Raptors can pose special challenges to captive propagators: small captive populations, incompatible and infertile pairs, and small semen volumes. Artificial insemination has been used successfully with many of these species (Gee and Temple 1978, Gee 1983, Weaver and Cade 1983). Wildlife managers need a reliable method for the preservation of semen to support these captive raptor propagation programs. Two raptor studies revealed some success in long-term preservation of Peregrine Falcon (*Falco peregrinus*; Parks et al. 1986) and American Kestrel (*F. sparverius*; Brock 1986) semen. Neither experimenter used dimethyl sulfoxide (DMSO) as the cryoprotectant. DMSO is superior to other cryoprotectants because it does not have to be removed from the semen prior to insemination (Sexton 1980, Gee et al. 1985). We have investigated the effect of cryopreservation on avian semen (Gee et al. 1985, Gee and Sexton 1990) and wanted to apply these techniques to raptors. In this study we attempted to determine the best DMSO concentration for cryopreservation of American Kestrel semen.

MATERIALS AND METHODS

Semen was collected on Monday, Wednesday, and Friday from 15 American Kestrel males during the normal breeding season in 1983–85 and stored frozen for 1–14 mo prior to use. Nine males were housed with females in 3 m wide, 15 m long, and 1.8 m high outdoor wire-mesh-covered pens. Males of the other six pairs were housed individually in similar pens with their females in the adjacent pens. Pens contained two shelter boxes, three hang-

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Table 1. Protocol for collection and cryopreservation of American Kestrel semen used in insemination trials.[a]

	Semen Dilution		
Trial	When Collected[b]	After Adding DMSO[c]	DMSO% (v/v)
1	1:1	1:2	4
2	1:3	1:7	4 and 6
3	1:0.5	1:1	6, 8 and 10

[a] Semen was collected at ambient temperatures from −12–+27°C, held at 1–5°C for <2 hr, equilibrated with DMSO for 10–15 min and frozen. Semen was frozen at 1°C/min from +5°–−20°C, at 50°C/min from −20°–−80°C, and at −160°C/min from −80°–−196°C. Semen was thawed in an ice bath at 0.5°C for 3 min. In each of three trials, 140 μl of semen was inseminated.
[b] BPSE (Sexton 1977) at 7.4 pH, 330 mmol/kg.
[c] DMSO diluted with semen extender before diluting semen.

ing perches, a water bowl, a feeding platform and a nest box (Porter and Wiemeyer 1970). Birds were fed Nebraska® Bird of Prey Diet (Central Nebraska Packing, Inc., North Platte, NE U.S.A.)[2] and day-old chickens on alternate days. The feeding platform and water bowl were cleaned daily. Birds were kept in these pens the year round. New individuals from our other colonies were introduced in February to replace birds injured or lost the previous year. After laying an infertile clutch of eggs, some of the single females (male in adjacent pen) were inseminated with thawed semen. Females were inseminated on Monday, Wednesday, and Friday and immediately after an egg was laid. Insemination following oviposition compensated for the days when an egg was present in the oviduct. Birds were not inseminated when an egg was present in the oviduct. For insemination, we chose females whose lay dates corresponded to times when adequate frozen semen pools were available.

We collected semen throughout the reproductive season from all males and, in all cases except those noted, diluted the semen as we collected it with Beltsville Poultry Semen Extender (BPSE; Sexton 1977). We modified the massage technique of Bird and Rehder (1981) to collect semen from several birds in the same container. We removed semen from the ventral lip of the cloaca with a small catheter with a Luer tip attached to a 1 ml syringe. A propipette, attached to the opposite end of the syringe, provided suction. The syringe barrel of the suction device was also used for measuring and diluting the semen.

An assistant held the kestrel in the palm of her hand while the operator stroked the abdomen with one hand and the base of the tail and ventral lip of the cloaca with the other hand. In the final step the operator forced the tail back with the heel of the hand and expressed the semen from the dorsal lip of the cloaca.

[2] Mention of this or any other commercial product does not constitute endorsement by the U.S. Government.

Semen was transferred to 1 ml collecting vials and placed in small (3 ml) test tubes that were partially submerged in an ice bath <5°C in the field, in transit, and upon return to the laboratory. Semen samples (5–10 μl) that contained motile sperm and little contamination were pooled. In seven pooled, undiluted semen samples, we measured osmolality and pH with a Wescor 5100B Vapor Pressure Indicator Osmometer and a Lazur® PHM-146 Micro pH Electrode according to manufacturer's instructions. In all semen samples we used progressive motility of sperm (Sexton and Gee 1978), degree of contamination, and concentration score (Gee and Temple 1978) to evaluate semen quality. We studied sperm morphology and obtained cell measurements in fresh semen (one drop of semen and one drop of stain under a 22 mm square coverslip) with a 1% eosin B or phloxine B stain, and in air dried smears stained with 1% phloxine B and 10% nigrosin stain, or in Bouin's fluid with eosin-nigrosin stain (Sigma HT10 1-32, Sigma Chemical Co., St. Louis, MO 63178 U.S.A.). We examined both the live wet mount and the air dried smear under a light microscope at 400×.

Sperm survival (progressive motility) in BPSE (7.4 pH, 330 mOs) and three variations (7.8 pH, 310 mOs; 7.0 pH, 310 mOs; and 7.5 pH, 280 mOs) were used to determine the most suitable semen extender for semen collection and cryopreservation. We estimated progressive motility (spermatozoa moving in a forward motion) on a scale from 0–100%. Samples for preservation were held in an ice bath (<5°C) for up to 2 hr before freezing. Semen samples were held in DMSO (4%, 6%, 8% or 10%; v/v) for 10–15 min before freezing. A 0.2 ml sample of diluted semen, equivalent to 3–4 ejaculates, was then placed in labeled plastic straws (0.5 ml Frenchstraw [Fiche de gontrola]; Edwards Agri-Sales Inc., Baraboo, WI 53913 U.S.A.), heat sealed, attached to a prenumbered semen cane, and frozen (Gee and Sexton 1979, Gee et al. 1985; Table 1). Frozen semen was transported in a portable liquid nitrogen tank and thawed for 3 min in an ice bath (0.5°C) immediately before use (Table 1). After thawing, we removed the semen from the straw with a small catheter and 1 ml syringe. The cloaca of the selected female American Kestrel was everted, exposing an everted vagina, the syringe was inserted 1–2 cm into the oviduct, the cloaca was allowed to return to the relaxed position with the syringe in place, and the semen was deposited. We everted the cloaca by holding the bird against our bodies. We applied a slow steady pressure to the abdomen while pressing the area around the cloaca with the thumbs and index fingers of both hands A 5–10 μl sample of the thawed semen was set aside at room temperature for semen quality evaluation (within 1 hr).

Fertility of eggs from birds inseminated with thawed semen and progressive motility of that semen were used to evaluate the effectiveness of cryopreservation. To determine fertility, eggs were candled at 14 d. Clear eggs were opened and examined for early death under a 7× dissecting microscope, and eggs with live embryos were returned to the nest.

Although the small data set makes statistical analysis tentative, we used the *t*-test to test the significance of differences between two sample means (Steel and Torrie 1960). We used the one-way classification analysis of vari-

ance where appropriate (Steel and Torrie 1960). Percentage values were sign transformed before analysis.

RESULTS AND DISCUSSION

The six female American Kestrels inseminated with thawed semen containing DMSO as cryoprotectant produced 48 eggs, 14 (29%) of which were fertile (Table 2). Of the 14 eggs incubated by females, 4 were broken and 6 of 10 eggs (60%) hatched. No fertile eggs were obtained from kestrels inseminated with thawed semen containing 4% DMSO (14 eggs). Six of 17 eggs (35%), 4 of 10 eggs (40%), and 4 of 7 eggs (57%) were fertile from kestrels inseminated with thawed semen containing 6%, 8% and 10% DMSO, respectively. Differences in fertility between kestrels inseminated with semen containing 6%, 8%, or 10% DMSO were not significant.

At least one-half of the kestrel sperm in seven freshly collected semen samples were progressively motile for up to 96 hr after storage at <5°C in BPSE at 7.0, 7.5, and 7.8 pH. No differences in motility were evident between extender pHs, but sperm concentration decreased in all samples. Therefore, the apparently high survival rate (50% motile cells) could have been an artifact if sperm were dying and disintegrating, as suggested by the decrease in concentration.

We were unable to determine percent live spermatozoa in thawed kestrel semen using conventional live-dead stains (Gee 1983). Stains such as eosin-nigrosin did not stain dead kestrel spermatozoa. In Peregrine Falcon semen, the opposite condition exists, eosin stains both live and dead cells (Hoolihan and Burnham 1985). Thawed semen contained fewer (57 ± 12%) progressively motile cells, a measure of sperm vitality (Smyth 1968), than semen prior to freezing (77 ± 9%; Fig. 1) with equivalent DMSO concentrations ($P \leq 0.001$). Also, thawed semen with 10% DMSO contained fewer progressively motile spermatozoa (44 ± 6%) than thawed semen with 6% (62 ± 10%) or 8% (61 ± 1%) DMSO ($P \leq 0.05$).

Individual ejaculates from American Kestrels are small (Bird and Lague 1977, Brock 1986), 10–20 µl in our study. The sperm concentration also is lower (Bird and Lague 1977) than in other birds (Gee 1983). Our American Kestrel semen yielded the lowest sperm score, 2 or less (Gee and Temple 1978), of any healthy bird examined in our laboratory. Most clean American Kestrel semen appears clear to slightly yellow. In addition to spermatozoa, kestrel semen usually contains clear, round bodies of various sizes, other debris, and, occasionally, a few squamous cells and erythrocytes.

Table 2. Reproductive success of female American Kestrels artificially inseminated with frozen and thawed semen containing 4, 6, 8, or 10% dimethylsulfoxide as the cryoprotectant in 1983–85.

	DIMETHYLSULFOXIDE			
	4%	6%	8%	10%
No. of kestrels	3	4	2	2
Eggs	14	17	10	7
Fertile eggs	0	6[a]	4[b]	4[b]
Infertile eggs	14	11	6	3
Eggs hatched	0	2	2	2

[a] Two eggs destroyed by kestrels.
[b] Egg destroyed by kestrels.

On smears stained in phloxine B (1%)-nigrosin (10%), the heads of kestrel spermatozoa varied in size and shape, but most (50%) were spherical (4–8 µm) in diameter. Sperm tails were of varying lengths (9–14 µm, one of 50 µm). The end of the tail was often broken, coiled or looped, even in the fresh semen sample. However, our method for making semen slides seemed to damage the spermatozoa. Our kestrel semen contained larger cells than those reported by Bird and Lague (1977; 2 µm × 4 µm) and by Brock (1986; 3.4 µm × 2.7 µm). We found and measured three classes of spermatozoa in the fixed semen smears: round (8.2 µm × 6.1 µm), elliptical (4.4 µm × 3.4 µm), and elongate (6.3 µm × 2.5 µm). Brock (1986) described the elliptical cell on slides stained with 1% glutaraldehyde in phosphate-buffered saline solution and stained with fast green FCF. It was the most common cell we observed (50% of the cells). However, the acrosomal cap and midpiece observed with the fast green technique were larger than those we observed on slides stained with phloxine.

We examined hundreds of slides and hundreds of cells per slide at a variety of light and filter settings and with phase contrast in an attempt to determine the morphology of the spermatozoa in fresh semen. Fresh semen stained with phloxine B (1%) produced the most consistent motility estimate because we could see the cells better against the pink background color than in a clear solution. Immotile cells, similar to the elliptical and round cells on prepared slides, were found in fresh semen stained with phloxine B. We

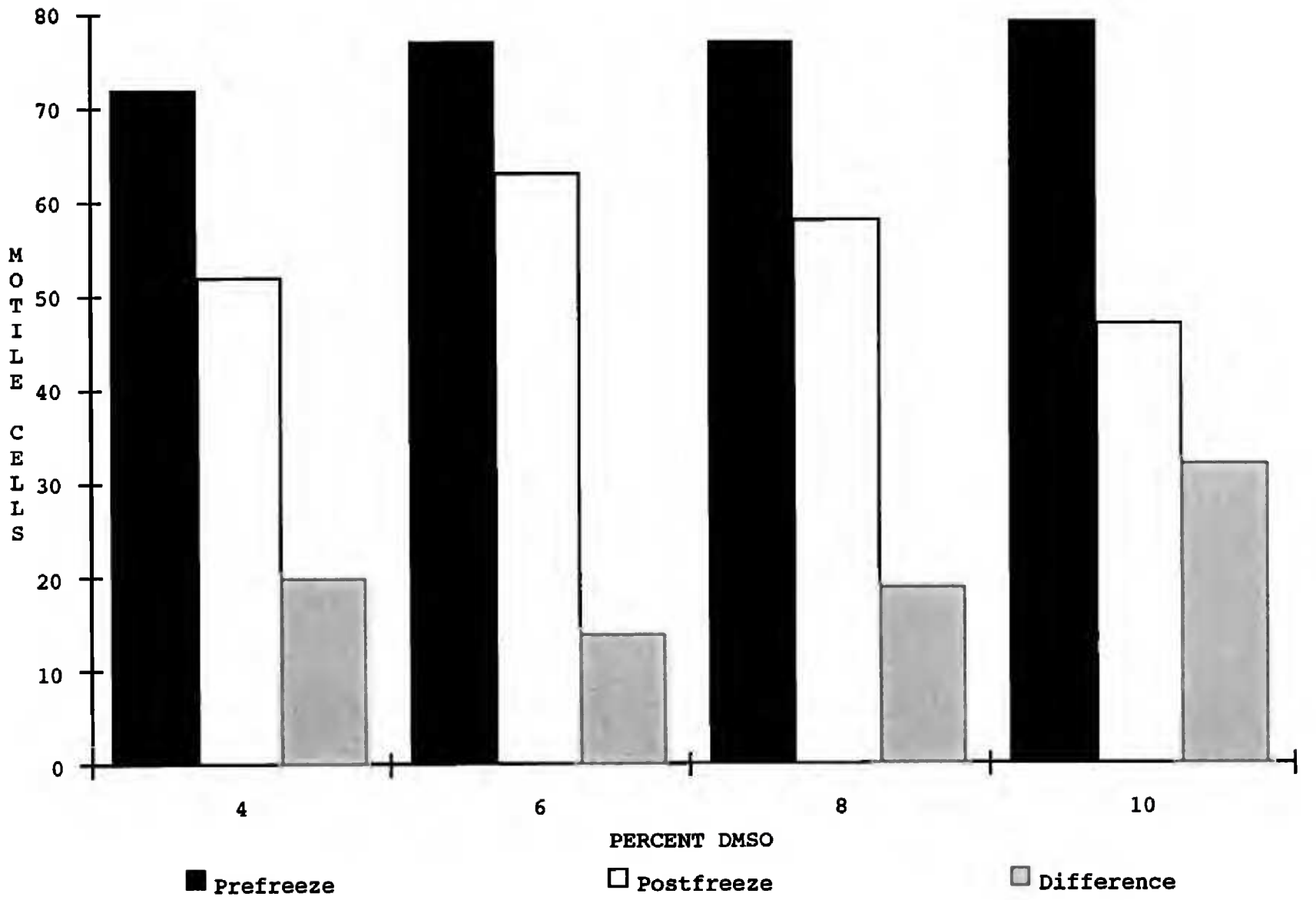

Figure 1. Progressive motility of kestrel semen cryopreserved in dimethylsulfoxide.

observed about 1% of these spherical cells lose their tails and, on occasion (about a dozen), burst, leaving only the red-stained acrosomal cap and midpiece. Our progressive motility estimates to evaluate treatments may have been in error because of the tendency of dead cells to burst. This may also explain why, in our evaluation of semen extenders, we observed a decrease in concentration after storage for 24 hr.

We found the spermatozoa of the American Kestrel unusual when compared to semen collected from other birds, in terms of shape, motility, cell number and live-dead staining characteristics. The round cell in kestrel semen resembled the droplet cell in crane semen (Gee and Temple 1978, Russman 1979). In all the semen examined for motility, we saw very few (4 or 5 per year) sauropsid cells, similar to those of Peregrine Falcons (scanning electron micrograph by W. Burnham pers. comm.) and in one case, a part of a sauropsid cell buried in the round cell. Sauropsid cells were rare but were always motile. In 281 species examined by McFarlane (1971) all avian sperm heads were cylindrical and tapering at each end. The gradual change from sauropsid to the elongated, markedly helical shape in passerines and other ultrastructural changes indicate phylogenetic relationships (McFarlane 1971). The true shape of kestrel spermatozoa, whether round as seen in fixed semen (fast green or phloxine slides) or sauropsid as seen on occasion in fresh semen, was not determined.

Semen with spherical sperm ranging from 2–14 μm long and 1–18 μm wide and propelled by a single 5–50 μm long tail produced fertile eggs. Although we tried to test for semen quality, the spermatozoa and semen characteristics of the American Kestrel restricted our study. We found the comparison of fertile egg production of limited usefulness because of the small number of birds and eggs in the study.

Fertile eggs and chicks can be produced by kestrels inseminated with thawed semen cryopreserved in

extender containing DMSO. Kestrels inseminated with thawed semen containing 6%, 8%, or 10% DMSO as the cryoprotectant produced fertile eggs and chicks. A 4% DMSO concentration (v/v) in the semen provided inadequate cryopreservation and 10% DMSO resulted in lower progressive motility than 6% or 8% DMSO.

LITERATURE CITED

BIRD, D.M. AND P.C. LAGUE. 1977. Semen production of the American Kestrel. *Can. J. Zool.* 55:1351–1358.

——— AND N.B. REHDER. 1981. The science of captive breeding of falcons. *Avicultural Magazine* 87(4):208–212.

BROCK, M.K. 1986. Cryopreservation of semen of the American Kestrel (*Falco sparverius*). M.Sc. thesis. Macdonald College of McGill University, Montreal, PQ Canada.

CADE, T.J. AND S.A. TEMPLE. 1977. The Cornell University falcon programme. Pages 353–369 *in* R.D. Chancellor [ED.], Report of Proceedings, World Conference on Birds of Prey, Vienna, Austria.

GEE, G.F. 1983. Avian artificial insemination and semen preservation. Pages 375–398 *in* A.C. Risser, Jr. and F.S. Todd [EDS.], Proceedings, Jean Delacour/International Foundation for the Conservation of Birds Symposium on Breeding Birds in Captivity, International Foundation for the Conservation of Birds, North Hollywood, CA U.S.A.

——— AND T.J. SEXTON. 1979. Artificial insemination of cranes with frozen semen. Pages 89–94 *in* J.C. Lewis [ED.], Proceedings of the 1978 Crane Workshop. Colorado State University Printing Service.

——— AND ———. 1990. Cryogenic preservation of semen from the Aleutian Canada Goose (*Branta canadensis leucoparia*). *Zoo Biology* 9:361–371.

——— AND S.A. TEMPLE. 1978. Artificial insemination for breeding non-domestic birds. *Symp. Zool. Soc. London* 43:51–72.

———, M.R. BAKST AND T.J. SEXTON. 1985. Cryogenic preservation of semen from the Greater Sandhill Crane. *J. Wildl. Manage.* 49:480–484.

HOOLIHAN, J. AND W. BURNHAM. 1985. Peregrine Falcon semen; a quantitative and qualitative examination. *Raptor Res.* 19:125–127.

KING, W.B. 1978. The Red Data Book. Aves, Vol. 2. International Union for the Conservation of Nature, Morgen, Switzerland.

MCFARLANE, R.W. 1971. Ultrastructure and phylogenetic significance of avian spermatozoa. Ph.D. thesis. University of Florida, Gainesville, FL U.S.A.

PARKS, J.E., W.R. HECK AND V. HARDASWICK. 1986 Cryopreservation of Peregrine Falcon semen and post-thaw dialysis to remove glycerol. *Raptor Res.* 20:15–20.

PORTER, R.D. AND S.N. WIEMEYER. 1970. Propagation of captive American Kestrels. *J. Wildl. Manage.* 34. 594–604.

RUSSMAN, S.E. 1979. Sperm morphology in the crane. M.Sc. thesis. University of Illinois, Urbana, IL U.S.A.

SEAGER, S., D. WILDT AND C. PLATZ. 1978. Artificial breeding of non-primates. *Symp. Zool. Soc. London* 43: 207–218.

SEXTON, T.J. 1977. A new poultry semen extender. Part 1. Effect of extension on the fertility of chicken semen. *Poult. Sci.* 56:1443–1446.

———. 1980. Optimal rates for cooling chicken semen from 5 to minus 196 Celsius. *Poult. Sci.* 59:2765–2770.

——— AND G.F. GEE. 1978. A comparative study on the cryogenic preservation of semen from the Sandhill Crane and the domestic fowls. *Symp. Zool. Soc. London* 43:89–95.

SMYTH, J.R., JR. 1968. Poultry. Pages 258–300 *in* E.J Perry [ED.], The artificial insemination of farm animals. Rutgers University Press, New Brunswick, NJ U.S.A.

STEEL, R.G.D. AND J.H. TORRIE. 1960. Principles and procedures of statistics. McGraw Hill Book Co., New York, NY U.S.A.

WEAVER, J.D. AND T.J. CADE. 1983. Falcon propagation: a manual on captive breeding. The Peregrine Fund, Inc., Ithaca, NY U.S.A.

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